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(57) Abstract

The present invention relates to peptides selectively immunoreactive with antibodies to *human immunodeficiency virus type 1 group 0*, nucleic acid sequences encoding these peptides, recombinant vector molecules, comprising these nucleic acid sequences, host cells transformed with the recombinant vector molecule, immunodiagnostic reagents comprising the peptides, a test kit for the detection of *HIV-1 group O* infections as well as a vaccine for the protection against *HIV-1 group O* infections. The peptides according to the invention comprise at least part of the V3-loop region of the HIV-ANT70 isolate, or a functional variant thereof. The peptides according to the invention comprise at least part of the amino acid sequence shown in SEQ ID No.:1 or a functional variant thereof. It has been found that the peptides according to the present invention are particularly suitable for diagnosing *HIV-1 group O* infected humans.

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Specific HIV-1 group O antigens

The present invention relates to peptides immunoreactive with antibodies to *human immunodeficiency virus type 1 group O* (to be referred to as *HIV-1 group O* hereinafter), nucleic acid sequences encoding these peptides, recombinant vector molecules, comprising these nucleic acid sequences, host cells transformed with the recombinant vector molecule, immunodiagnostic reagents comprising the peptides, a test kit for the detection of *HIV-1 group O* infections as well as a vaccine for the protection against *HIV-1 group O* infections.

Substantial progress has been made in our understanding of the acquired immunodeficiency syndrome or AIDS. The principal causative agent has been demonstrated to be a non-transforming retrovirus with a tropism for T4 helper inducer lymphocytes (Dalgleish et al., 1984; Maddon et al., 1986) and it has been estimated that millions of people world-wide have already been infected. Infection with its virus leads, at least in a significant percentage of cases to a progressive depletion of the T4 lymphocyte population with a concomitant increasing susceptibility to the opportunistic infections which are characteristic of the disease. Epidemiological studies indicate that human immunodeficiency virus type 1 (HIV-1), the etiological agent responsible for the majority of AIDS cases and which is currently the most widely disseminated HIV, probably had its origins in Central Africa (Wong-Staal et al., 1985). The discovery of this virus did not necessarily imply the existence of other types of human immunodeficiency viruses. Nevertheless, a second group of human immunodeficiency-associated retroviruses, human immunodeficiency virus type 2 (HIV-2) was identified in West Africa (Clavel et al., 1986; Albert et al., 1987). Other similar but not identical, retroviruses have also been isolated from simian sources (simian immunodeficiency virus, SIV) such as African green monkeys (Kanki et al., Lancet, 1985; Kanki et al., Science, 1985) and macaques (Daniel et al., 1985; Chakrabarti et al., 1987). The simian isolates have been shown to be genetically more closely related to HIV-2 than HIV-1 but are nevertheless distinct (Franchini et al., 1987).

One characteristic of human immunodeficiency viruses which complicates their comparison is their genetic variability, genetic variants arise spontaneously and with high frequency. A comparison of various HIV-1 isolates revealed that some regions of the genome are highly variable while others are reasonably well conserved (Benn et al., 1985; Hahn et al., 1986; Magasiny et al., 1986; Alizon et al., 1985; Starcich et al., 1986; Willey et al., 1986). Similar polymorphisms have also been observed for HIV-2 (Clavel et al., 1986). The regions with the greatest genetic stability are presumably those regions coding for the regions of viral proteins which are structurally or enzymatically essential. The viral genes with the greatest overall genetic stability are the gag and pol genes, while some regions of the env gene and the genes coding for regulatory proteins such as art, tat, sor and 3 orf exhibit a high degree of variability. Some of the major structural features of the gag and pol gene products are apparently shared not only by all of the variants of a particular HIV type, but have, at least to some extent, been conserved between virus types. Antiserum produced against HIV-1 cross-reacts with the gag and pol gene products of HIV-2, albeit with a lower affinity than for the corresponding HIV-1 gene products. However, in spite of the

demonstrable immunological cross-reaction, at the nucleic acid level there is little sequence homology and no significant hybridization between these two viruses can be detected except under very low stringency conditions (Clavel et al., 1986).

Nucleotide sequence analysis of a vast number of different HIV-1 isolates showed that this virus type can be divided into at least six distinct env gene subtypes, designated A to F (Myers, 1994). Each of these subtypes is equally distant to the others in a phylogenetic pattern recognized as a 'star phylogeny'.

More recently, two distant HIV-1 isolates, HIV-1 ANT70 and HIV-1 MVP5180, obtained from patients living in Cameroon, in west central Africa, have been described and molecularly characterized (De Leys et al., 1990, Gürtler et al., 1994, Vanden Heasevelde et al., 1994). These two closely related viruses are clearly more distant to the other six subtypes than any previously recognized HIV-1 isolate and have been proposed to be part of a separate subtype termed O (the letter O standing for outlier). However, the diversity of isolates within this group is unknown.
Its spread outside is recently documented in a report of a third isolate in which the isolation and env gene sequence, termed HIV-1 VAU, obtained from a French woman who had never travelled outside Europe and who died of AIDS in 1992 is described (Charneau et al, 1994). According to its envelope sequence, HIV-1 VAU is closely related to the two recently characterized cameroonian viruses HIV-1 ANT70 and HIV-1 MVP5180. Phylogenetic analysis of env sequences reveals that the three viruses are approximately equidistant to each other and that their mutual divergence is comparable to that found between other subtypes of HIV-1. Therefore, these three viruses appear to constitute a separate group, which is called the HIV-1 group O, as opposed to the main cluster of HIV-1 group M (the letter M standing for major).
The isolation of HIV-1 VAU from this French patient also indicates that at least some spread of HIV-1 group O has already occurred outside of Africa.

Envelope gene diversity

The high genetic diversity in env gene sequences of HIV-1 complicates both serodiagnosis and the development of an effective vaccine for AIDS. Numerous variant strains have been obtained from different geographic regions. At present, the HIV-1 group M viruses consist of six distinct genotypic subtypes, based on the sequences of the env gene (Myers et al., 1993; Myers, 1994; Louwagie et al., 1993; Janssens et al., 1994). The HIV-1 subtypes are approx. 30% different from one another in their envelope coding sequences and 14% different from one another in their gag coding sequences. The difference in envelope sequences determined by sequence analysis and heteroduplex mobility assays, is used to classify (genotype) the isolated viruses in different subtypes.
Most of the sequence variability found in gp120 is clustered in five hypervariable domains (V1-V5) (Willey et al., 1986; Modrow et al., 1987; Simmonds et al., 1990). The hypervariable domains are flanked by cysteine residues which greatly contribute to the overall structure of the envelope protein by forming loop-structures. The third variable region (V3-loop) is a major target of vaccine research, because it contains an important neutralization site, and has been shown to bind and elicit isolate-specific

neutralizing antibodies (Goudsmit et al., 1988; Matsuhita et al., 1988; Parker et al., 1988; Rusche et al., 1988). The V3-loop of the envelope protein comprises also B- and T-cell epitopes and plays a role in a variety of biological processes of virus replication such as attachment, penetration, viral fusion and syncytium formation (Freed et al., 1991; Groenink et al., 1992). Binding studies with human anti-HIV antibodies revealed that binding titers against a 15-mer V3 peptide do not predict binding titers against a 36-mer cyclic V3 peptide, or against native or denatured gp120 (Moore et al., 1993). The base regions of the V3-loop are not exposed on native gp120; consequently long peptides can lead to false-positive binding. Short peptides, on the other hand, may lead to false negatives (Moore, 1993).

The comparison between the HIV-1 group O and HIV-1 group M envelope amino-acid sequence showed approximately 50% identity whereas the inter-group O envelope identity is approx. 70%. Compared with the consensus V3 sequences of HIV-1 group M subtypes A, B, and D, the V3 sequence of HIV-1 group O isolate ANT70 differs in 60 to 70 % of the amino acids (Vanden Haesevelde et al., 1994). In most HIV-1 isolates worldwide, the central region of this domain contains a conserved sequence (GPGQ; Myers et al., 1993) which is not found in the HIV-1 group O isolates. The central part (tip) of the V3-loop region has been shown to be much less susceptible to mutations than are the flanking regions in strains isolated from sexual partners or even from the same individual at different stages of disease (Holmes et al., 1992; Wolfs et al., 1990; 1992). The amino-acid sequences at the tip of the V3-loop of the published HIV-1 group O viruses are very different from all other V3 loops reported sofar i.e. GPMAWYS (HIV-ANT70, HIV-VAU) and GRMRWRS (HIV-MVP5180) (Janssens et al., 1994; Charneau et al., 1994).

The high variability in the envelope sequences is also reflected by the lack of sensitivity for the detection of antibodies in HIV-1 group O infected patients by certain screening assays (Simon et al., 1994; Schable et al., 1994). All assays based on synthetic peptides or recombinant antigens of HIV-1 group M viruses failed to detect at least one or more of the group O infections. Assays based on whole-virus lysates performed better (Schable et al., 1994). Validation of the HIV infection on the basis of western blot and immunofluorescence reactivity is not always conclusive. Most HIV-1 group O serum samples show a weak or absent gp160, gp120, and monomeric transmembrane glycoprotein gp41 reactivity. Furthermore, mostly there are no crossed reactions with the precursor of gag p55. Inversely, a constant reactivity has been observed for protein p31, corresponding to the pol gene product (Simon et al., 1994). Therefore, the western blot assay currently used as a reliable confirmation assay for HIV infection has become questionable with the discovery of HIV-1 group O viruses.

Accordingly, the invention relates to peptides comprising a specific region of the V3-loop region of HIV-1 isolates which is solely reactive with antibodies of HIV-1 group O viruses and is <u>not</u> reactive with antibodies of HIV-1 group M specimens. Therefore, said peptides can be applied as antigen in diagnostic assays to confirm an HIV-1 group O infection. In addition said peptides can be applied in genotyping

studies in order to discriminate between HIV-1 group M and group O virus infections.

The present invention provides new peptides, selectively immunoreactive with antibodies to *HIV-1 group O*, that can be used in diagnosing *HIV-1 group O* infections in humans.
With the term "selectively" is meant that the peptides do not cross-react with HIV-1 group M sera.
In particular the present invention provides peptides, selectively immunoreactive with antibodies to *HIV-1 group O*, comprising at least part of the amino acid sequence of a linearized V3-loop region or a functional variant thereof.
A preferred embodiment of the present invention are peptides comprising at least part of the V3-loop region of the HIV-ANT70 isolate, or a functional variant thereof.
Another preferred embodiment of the present invention are peptides comprising at least part of the amino acid sequences as shown in SEQ. ID No. 1, or a functional variant thereof.
Another preferred embodiment of the present invention are polypeptides comprising polymeric forms of said peptides.

The term "peptide" as used herein refers to a molecular chain of amino acids with a biological activity, and does not refer to a specific length of the product. Thus inter alia, proteins, oligopeptides, polypeptides and fusion-peptides as well as fusion-proteins are included.
The term "polypeptide" refers to dimeric, trimeric, . . . polymeric forms of any length of a peptide according to the present invention.
The term "functional variants" as used herein means, for example, acid addition salts, amides, esters, and specifically C-terminal esters, and N-acyl derivatives of the peptides according to the invention are also considered part of the present invention
Also included are peptides which are modified in vivo or in vitro, for example by glycosylation, amidation, carboxylation or phosphorylation. It will be understood that for the particular proteins or polypeptides embraced herein, natural variations can also exist. These variations may be demonstrated by (an) amino acid difference(s) in the overall sequence or by deletions, substitutions, insertions, inversions or additions of (an) amino acid(s) in said sequence. Amino acid substitutions from which can be expected that they do not essentially alter biological and immunological activities, have been described. Amino acid replacements or conservative replacements between related amino acids or replacements which have occurred frequently in evolution are, inter alia Ser/Ala, Ser/Gly, Asp/Gly, Asp/Asn, Ile/Val (see Dayhof, M.D., Atla of protein sequences and structure, Nat. Biomed. Res. Found., Washington D.C., 1978, vol. 5, suppl. 3).
Also the synthesis of peptides which are closely related to the peptide as SEQ.ID 1 but comprising non-natural amino acids is known in this field of research. Peptides synthesized by these non-natural amino acid replacement techniques will be also part of this invention.

The term "at least part of the amino acid sequence" as used herein means an amino acid sequence comprising a subsequence of a peptide of the invention. Said parts or fragments are peptides comprising at least one antigenic determinant of the amino acid sequences as shown in SEQ. ID No.: 1 which specifically reacts with antibodies from HIV-1 group O patients only and do not cross-react with antibodies from HIV-1 group M patients. Fragments can inter alia be produced by enzymatic cleavage of precursor molecules, using restriction endonucleases for the DNA and proteases for the polypeptides. Other methods include chemical synthesis of the fragments or the expression of peptide fragments by DNA fragments.

Suitable antigenic fragments of a peptide according to the invention containing (an) epitope(s) can be found by means of the method described in Patent Application WO 86/06487, (Geysen, H.M. et al. (Proc. Natl. Acad. Sci. 81, 3998-4002, 1984;, Geysen, H.M. et al. J. Immunol. Meth. 102, 259-274, 1987) based on the so-called pepscan method, wherein a series of partially overlapping peptides corresponding with partial sequences of the complete polypeptide under consideration, are synthesized and their reactivity with antibodies is investigated.

The minimal antigenic fragments of the epitopes can be found by synthesizing a series of partially overlapping peptides consisting of a gradually increase in amino acids per scan. The minimal immunoreactive core-structure of the epitope can be used to synthesize polymeric synthetic peptides on branched lysine molecules. Application of these branched peptides in a diagnostic assay results in an increase in immunoreactive sites for antibodies whereof the sensitivity of the assay can significantly be increased.

The peptides according to the invention comprising the amino acid sequence as shown in SEQ. ID No. 1 are recognized by *HIV-1 group O* antibodies of the IgG- as well as IgM-class, and therefore are suitable diagnostic markers for *HIV-1 group O* infections.

In SEQ. ID No.: 1 and 3 the amino acid sequences are given for peptides with a (calculated) molecular weight of approximately 4.2 kD and 3.6 kD, respectively. Peptides comprising these sequences, or parts thereof, are part of the present invention.

The preparation of the peptides or fragments thereof according to the invention is effected by means of one of the known organic chemical methods for peptide synthesis or with the aid of recombinant DNA techniques. This latter method involves the preparation of the desired peptide by means of bringing to expression a recombinant polynucleotide with a polynucleotide sequence which is coding for one or more of the peptides in question in a suitable micro-organism as host.

The organic chemical methods for peptide synthesis are considered to include the coupling of the required amino acids by means of condensation reaction, either in homogeneous phase or with the aid of a so-called solid phase.

The condensation reaction can be carried out as follows:

a) condensation of a compound (amino acid, peptide) with a free carboxyl group and protected other reactive groups with a compound (amino acid, peptide) with a free

amino group and protected other reactive groups, in the presence of a condensation agent;
b) condensation of a compound (amino acid, peptide) with an activated carboxyl group and free or protected other reaction groups with a compound (amino acid, peptide) with a free amino group and free or protected other reactive groups.
Activation of the carboxyl group can take place, inter alia, by converting the carboxyl group to an acid halide, azide, anhydride, imidazolide or an activated ester, such as the N-hydroxy-succinimide, N-hydroxy-benzotriazole or p-nitrophenyl ester.

The most common methods for the above condensation reactions are: the carbodiimide method, the azide method, the mixed anhydride method and the method using activated esters, such as described in The Peptides, Analysis, Synthesis, Biology Vol. 1-3 (Ed. Gross, E. and Meienhofer, J.) 1979, 1980, 1981 (Academic Press, Inc.).

Preparation of suitable fragments of above-mentioned peptides according to the invention using the "solid Phase" is for instance described in J Amer Chem. Soc. 85, 2149 (1963) and Int J. Peptide Protein Res 35, 161-214 (1990)

As already indicated above, the peptides according to the invention can likewise be prepared with the aid of recombinant DNA techniques. For example, the peptides according to the invention can be incorporated in a repeating sequence ("in tandem") or can be prepared as a constituent of a (much larger) protein or polypeptide. This type of peptides therefore likewise falls within the scope of the invention.

For this purpose, as a constituent of a recombinant DNA, a nucleic acid sequence is used which codes for a peptide according to the invention and which, furthermore, is substantially free from nucleic acid segments, which in the naturally occurring *HIV-1 group O* genome flank the nucleic acid sequence indicated above
This latter method involves the preparation of the desired peptide by means of bringing to expression a recombinant polynucleotide with a nucleic acid sequence which is coding for one or more of the peptides in question in a suitable micro-organism as host.

The invention further encompasses nucleic acid sequences encoding the peptides according to the invention and nucleic acid sequences containing at least part of the DNA sequence shown in SEQ. ID No.: 2. The nucleic acid sequence as shown in SEQ. ID No.: 2 encodes the peptide as shown in SEQ. ID. No.: 1, while the nucleic acid sequence as shown in SEQ. ID No.: 4 encodes the peptide as shown in SEQ. ID No.: 3.
The invention also comprises (a) host cell(s) transformed or transfected with a nucleic acid sequence or recombinant expression vector molecule, capable of producing the peptides according to the invention by expression of the corresponding nucleic acid sequence.

"Nucleic acid sequence" as used herein refers to a polymeric form of nucleotides of any length, both to ribonucleic acid sequences and to deoxy ribonucleic acid

sequences. In principle, this term refers to the primary structure of the molecule. Thus, this term includes double and single stranded DNA, as well as double and single stranded RNA, and modifications thereof.

A nucleic acid sequence according to the present invention can be ligated to various replication effecting DNA sequences with which it is not associated or linked in nature resulting in a so called recombinant vector molecule which can be used for the transformation of or transfection into a suitable host. Useful recombinant vector molecules, are preferably derived from, for example plasmids, bacteriophages, cosmids or viruses.

Specific vectors or cloning vehicles which can be used to clone nucleic acid sequences according to the invention are known in the art and include inter alia plasmid vectors such as pBR322, the various pUC, pGEM and Bluescript plasmids, bacteriophages, e.g. lambda gt-Wes, Charon 28 and the M13 derived phages or viral vectors such as SV40, adenovirus, Semliki Forest Virus, vaccinia virus, Herpes viruses or polyoma virus (see also Rodriquez, R.L. and D.T. Denhardt, ed., Vectors: A survey of molecular cloning vectors and theirs uses, Butterworths, 1988; Lenstra, J.A. et al., Arch. Virol. 110, 1-24, 1990). The methods to be used for the construction of a recombinant vector molecule according to the invention are known to those of ordinarily skill in the art and are inter alia set forth in Maniatis, T. et al. (Molecular Cloning A Laboratory Manual, second edition; Cold Spring Harbor Laboratory, 1989).

For example, the insertion of the nucleic acid sequence according to the invention into a cloning vector can easily be achieved when both the genes and the desired cloning vehicle have been cut with or either one of them has been digested with the same restriction enzyme(s) as complementary DNA termini are thereby produced The recombinant vector molecules may additionally contain one or more marker activities that may be used to select for desired transformants, such as ampicillin and tetracycline resistance in pBR322, as for example ampicillin resistance and Â-peptide of ß-galactosidase in pUC8.

A suitable host cell is a micro-organism or cell which can be transformed by a nucleic acid sequence encoding a polypeptide or by recombinant vector molecule comprising such a nucleic acid sequence and which can if desired be used to express said polypeptide encoded by said nucleic acid sequence. The host cell can be of procaryotic origin, e.g. bacteria such as Escherichia coli, Bacillus subtilis and Pseudomonas species; or of eucaryotic origin such as yeasts, e.g. Saccaromyces cerevisiae or higher eucaryotic cells such as insect, plant or mammalian cells, including HeLa cells and Chinese hamster ovary (CHO) cells.

Information with respect to the cloning and expression of the nucleic acid sequence of the present invention in eucaryotic cloning systems can be found in Esser, K. et al. (Plasmids of Eucaryotes, Springer-Verlag, 1986).

In general, prokaryotes are preferred for the construction of the recombinant vector molecules useful in the invention. For expression nucleic acid sequences of the present invention are introduced into an expression vector, i.e. said sequences are operably linked to expression control sequences. Such control sequences may comprise promoters, enhancers, operators, inducers, ribosome binding sites etc.

Therefore, the present invention provides a recombinant vector molecule comprising a nucleic acid sequence encoding the peptides identified above operably linked to expression control sequences, capable of expressing the DNA sequences contained therein in (a) transformed host cell(s).

It should, of course, be understood that the nucleotide sequences inserted at the selected site of the cloning vector may include only a fragment of the complete nucleic acid sequence encoding the peptides according to the invention as long as the transformed or transfected host will produce a polypeptide having at least one or more antigenic determinants.

In order to purify the expressed polypeptides produced as described above, host cells transformed with a recombinant vector according to the invention are cultured in an adequate volume and the polypeptides produced are isolated from such cells or from the medium if the protein is excreted. Polypeptides excreted into the medium can be isolated and purified by standard techniques, e.g. salt fractionation, centrifugation, ultrafiltration, chromatography, gel filtration or immunoaffinity chromatography, whereas intra cellular polypeptides can be isolated by first collecting said cells, disrupting the cells, for example by sonication or by other mechanically disruptive means such as French press followed by separation of the polypeptides from the other intracellular components and forming isolated polypeptides. Cell disruption could also be accomplished by chemical (e.g. EDTA or detergents such as Triton X114) or enzymatic means such as lysozyme digestion.

Antibodies, immunoreactive with a peptide according to the invention are also part of the present invention.
The peptides or fragments thereof prepared and described above are used to produce antibodies, both polyclonal and monoclonal Monoclonal antibodies directed against peptides according to the invention can be readily produced by one skilled in the art

The preparation of cell lines producing monoclonal antibodies may occur by, for example, the Köhler and Milstein technique (Köhler and Milstein devised the techniques that resulted in the formation monoclonal antibody-producing hybridomas (G. Köhler and C. Milstein, 1975, Nature 256:495; 1976, Eur. J. Immunol. 6:511-519)), transformation technique of B-lymphocytes with a fusion partner being either a human or a mouse-human hybrid myeloma cell line, or a direct fusion of an EBV-tranformed B cell line with said myeloma cell lines, or a direct fusion by electro-fusion techniques (co-owned and co-pending patent application EP 0 488 470).

An immunochemical reagent comprising one or more peptides or antibodies according to the invention is also part of the present invention.

The term "immunochemical reagent" according to the invention usually consists of one more peptides according to the invention and a suitable support or a labelling substance. Supports which can be used are, for example, the inner wall of a microtest well or a cuvette, a tube or capillary, a membrane, filter, test strip or the surface of a particle such as, for example, a latex particle, a ceramic particle, a ceramic

magnetizable particle, an erythrocyte, a dye sol, a metal sol or metal compound as sol particle, a carrier protein such as BSA or KLH.
Labelling substances which can be used are, inter alia, a radioactive isotope, a fluorescent compound, an enzyme, a dye sol, a metal sol or metal compound as sol particle.

In a method for the detection of antibodies directed against *HIV-1 group O* in a sample, an immunochemical reagent according to the invention is brought into contact with the sample. After which, the presence of immune complexes formed between the peptide and antibodies in the sample is detected and by this detection the presence of *HIV-1 group O* antibodies in the sample is known and can be determined quantitatively.

Depending on the nature and further characteristics of the immunochemical reagent the immunochemical reaction that takes place is a so called sandwich reaction, an agglutination reaction, a competition reaction or an inhibition reaction. Also known one-step and multiple-step reactions are part of the present invention.

A particularly suitable method for the detection of *HIV-1 group O* in a sample is based on a competition reaction between a peptide according to the invention provided with a labelling substance and a *HIV-1 group O* antigen (present in the sample) whereby the peptide and the antigen are competing with the antibody directed against *HIV-1 group O* attached to a solid support.

An antibody according to the invention may also be brought into contact with a sample whereafter the presence of immune complexes formed is detected which is a measure for the presence of *HIV-1 group O* in the sample.

A diagnostic test kit according to the invention may comprise as an essential constituent an immunochemical reagent as described above. For carrying out a sandwich reaction, for the detection of *HIV-1 group O* antibodies the test kit may comprise, for example, the peptide according to the invention coated directly or via a carrier-protein, i.e. BSA, to a solid support, for example the inner wall of a microtest well or a particle or a sol, and either a labelled peptide according to the invention or a labelled anti-antibody.
For carrying out a competition reaction, the test kit may comprise a peptide according to the invention coated to a solid support, and a labelled antibody directed against *HIV-1 group O* preferably a monoclonal antibody directed against said peptide.

In an agglutination reaction the test kit comprises an immunochemical reagent which may comprise a peptide according to the invention coated to particles or sols.
Another embodiment of a test kit is, for example, the use of a labelled peptide according to the invention as immunochemical reagent in a competition reaction with a *HIV-1 group O* antigen to be detected for a binding site on the antibody directed against *HIV-1 group O*, which is coated to a solid support.

It is within the scope of this invention to use the new nucleic acid sequences according to the invention as the basis of a test to detect *HIV-1 group O* by a nucleic acid amplification technique for instance the polymerase chain reaction (PCR) or the nucleic acid sequence based amplification (NASBA), as described in EP 201,814 and EP 329,822, respectively A method for the amplification and the detection of a *HIV-1 group O* nucleic acid sequence in a sample using at least one nucleic acid sequence or fragment thereof according to the invention primer(s) in order to perform a nucleic acid amplification of said *HIV-1 group O* nucleic acid sequence and to detect the amplified sequence is also part of the present invention. Part of the invention is also a test amplification technique, said kit containing at least a set of primers corresponding to at least a part of the nucleotide sequences according to the invention.

Vaccines for the protection against *HIV-1 group O* are also part of the present invention. These vaccines comprise a peptide according to the invention or a polypeptide according to the invention, together with a pharmaceutical acceptable carrier.
The vaccine according to the invention can be administered in a conventional active immunization scheme: single or repeated administration in a manner compatible with the dosage formulation and in such amount as will be prophylactically effective, i.e the amount of immunizing antigen that will induce immunity against challenge by *HIV-1 group O*. Immunity is defined as the induction of a significant level of protection in a population after vaccination compared to an unvaccinated group.
The administration of the vaccine can be done, e.g. intradermally, subcutaneously, intramuscularly, intraperitoneally, intra-venously, orally or intranasally.
Additionally the vaccine may also contain an aqueous medium or a water containing suspension, often mixed with other constituents, e.g. in order to increase the activity and/or shelf life. These constituents may be salts, pH buffers, stabilizers (such as skimmed milk or casein hydrolysate), emulsifiers, adjuvants to improve the immune response (e.g. oils, muramyl dipeptide, aluminiumhydroxide, saponin, polyanions and amphipatic substances) and preservatives.

Also part of the present invention is the use of a peptide according the present invention for the selective detection of antibodies to human immunodeficiency virus type 1 (HIV-1) group O.

Brief description of the figures:

Figure 1: HPLC profile of V3-ANT70 peptide.
Figure 2: HPLC profile of V3-MVP peptide:

The invention is further exemplified by the following examples:

Example 1

Synthesis and purification of V3-loop region peptides

The V3-loop region peptides of the HIV-1 group O isolates MVP5180 and ANT70, comprising the amino acid sequences:
NH2-CIREGIAEVQDIYTGPMRWRSMTLKRSNNTSPRSRV-COOH (MW 4.209 kD; SEQ.ID. 3) and
NH2-CERPQIDIQEMRIGPMAWYSMGIGGTAGNSSRA-COOH (MW 3.583 kD; SEQ.ID. 1), respectively, were synthesized sequentially on a solid support via the Fmoc/tBu chemistry. The peptides will be referred to as V3-MVP and V3-ANT70. The solid support is of the Rink-type, which automatically yields a C-terminally amidated peptide. During solid phase peptide synthesis the amino acid side chains were protected with acid-labile protecting groups: the _-amino group of lysine with Boc, the _-guanodino group of arginine with Pmc, the _-carboxyl group of glutamic acid and the ß-carboxyl group of aspartic acid with OtBu, the _-amide group of glutamine and the ß-amide group of asparagine with Trt, and the ß-hydroxyl group of serine with tBu. All reactants were dissolved in NMP. The cleavage of the Fmoc groups was carried out with 25% (vol/vol) piperidine in NMP during at least two consecutive cycles of 3 minutes. Coupling of the first amino acid derivative (Fmoc-Ala-OH, 1 mmol) was performed (in situ activation with HBTU, HOBt, and DIEA). After coupling of each amino acid derivative (at least 30 minutes), no check for completion of the acylation reaction was carried out. The acylation reaction was followed by a capping-step with acetic anhydride. After the identical, single coupling acylation cycles the fully protected peptides were cleaved from the resin during a 2-hr reaction with 3.5% (vol/vol) ethanedithiol, 5% (vol/vol) thioanisole, 2.5% (vol/vol) water, and 2% (vol/vol) anisole in trifluoroacetic acid followed by precipitation in diethyl ether. The crude peptides were dissolved in water/acetonitril (2.1). After a freeze drying time of 72 hours weights of V3-ANT70 and V3-MVP crude peptides were 75 mg (33% recovery) and 53 mg (33% recovery), respectively. The synthesis of the peptides was carried out on an Applied Biosystems Inc. (division of Perkin Elmer) 433 peptide synthesizer, using standard FastMoc® 0.25 mmol procedures with conductivity monitoring and feedback option.
The HPLC-patterns of the crude V3-ANT70 and V3-MVP peptides (column 4 x 200 Superpack prepS 5µ; mobile phase A: 0.1% TFA/water, B:100% acetonitrile/water + 0.1% TFA; gradient t= 3 min. 0% B, t= 33 min. 75% B, detection at 206 nm; flow 1 ml/min) show a main peak at 21.2 minutes with a peak area of 27%, and 19.9 minutes with a peak area of 38%, respectively (Figure 1 and 2).

Example 2

Coupling peptides to BSA

Peptides were coupled to bovine serum albumine (BSA) on equal molar ratio through glutaraldehyde linkaging. Briefly, 8.5 mg of V3-ANT70 peptide and 7.5 mg of V3-MVP peptide were dissolved in 475 µl 100 mM sodium phosphate buffer (pH 7.0) and added to 475 µl BSA-buffer (~20 g BSA dissolved in 100 mM phosphate buffer,

pH 7.0). Subsequently, 475 µl 0.02 mol/l glutaraldehyde solution in phosphate buffer (pH 7.0) was added to the protein mixture. The peptide-BSA mixtures were incubated for 18 hours at room temperature after which 142.5 µl 10x concentrated stabilizing buffer (phosphate buffer supplemented with saccharose, glycine and sodiumborohydrate) was added. The peptide-BSA mixtures, designated V3-ANT70/BSA and V3-MVP/BSA were incubated for 1 hour at room temperature, aliquoted and stored at -70 °C.

Example 3

Coating experiments

Microtiter plates were coated overnight at room temperature with 5-fold dilutions of 135 µl V3-ANT70, V3-ANT70/BSA and V3-MVP peptides in coating-buffer (carbonate-buffer, pH 9.6). After removal of the unbound antigens, plates were postcoated. After drying, the plates were stored at 4 °C until use. The optimum coat conditions were determined by incubating the coated plates with 1/100 dilution of the following defined human serum samples: normal serum (NHS), HIV-1 group M, HIV-2 and two HIV-1 group O samples (100 µl/well). The two HIV-1 group O serum samples designated **Y42** and **NAAH**, were confirmed to be group O samples by sequence analysis and were kindly provided by Dr. Zekeng (Yaoundé) and Dr. Simon (Paris), respectively. Following 30 min. incubation at 37 °C, wells were washed with phosphate-buffered saline-Tween solution (PBST) and incubated with 100 µl horse-radish conjugated mouse anti-human IgG monoclonal antibodies After 30 min. incubation at 37 °C, wells were washed with PBST and the antigen-antibody complexes were detected by the addition of 100 µl TMB as substrate. After 30 minutes incubation at room temperature the reaction was stopped by adding 100 µl 1M H_2SO_4 to the wells. Optical densities were measured at 450 nm. These studies revealed that both sera derived from HIV-1 group O infected patients were immunoreactive with the V3-ANT70 and V3-ANT70/BSA antigens (Table 1). However, irrespective of the antigen coat concentration, serum sample Y42 did not react with the V3-MVP antigen. One other serum sample collected from a confirmed HIV-1 group O infected patient (Dür) also did not recognise the V3-MVP peptide (data not shown). As shown in Table 1, none of HIV-1 group M and HIV-2 antibodies react with the V3-ANT70 peptide. These studies show that an HIV-1 group O specific assay can be developed solely based on the V3-ANT70 peptide coated on the solid phase either as free-peptide or coupled to a carrier protein i.e BSA. High-titered human anti-HIV-1 group M serum samples were tested on cross-reactivity with the V3-ANT70 peptide. As shown in Table 2, none of the serum samples tested reacted with the V3-ANT70/BSA peptide

Tabel 1. Immunoreactivity of human serum samples with V3-ANT70 and V3-MVP peptides coated at different concentrations.

	Relative concentration of coated antigens (OD450)								
	V3-ANT70/BSA			V3-ANT70			V3-MVP		
Serum sample	25x	5x	1x	1/5x	5x	1x	1/5x	5x	1x	1/5x
NHS (nr.1)	0.052	0.055	0.062	0.055	0.053	0.050	0.055	0.053	0.053	0.058
NHS (nr.2)	0.047	0.054	0.059	0.057	0.047	0.045	0.058	0.051	0.053	0.060
HIV-1 "M" pos.(nr.1)	0.070	0.106	0.094	0.075	0.072	0.066	0.078	0.075	0.071	0.075
HIV-1 "M" pos.(nr.2)	0.048	0.072	0.058	0.051	0.061	0.056	0.052	0.060	0.053	0.051
HIV-2 pos. (nr.1)	0.042	0.059	0.050	0.042	0.040	0.038	0.043	0.042	0.044	0.046
HIV-2 pos. (nr.2)	0.043	0.067	0.051	0.044	0.045	0.039	0.043	0.045	0.047	0.051
HIV-1 "O" pos. (Y42)	0.449	1.356	0.286	0.068	0.398	0.144	0.053	0.052	0.048	0.050
HIV-1 "O" pos. (NAAH)	2.132	2.525	2.340	1.366	2.389	2.067	1.657	1.263	0.216	0.178

Tabel 2. Immunoreactivity of high-titered HIV positive human sera with the V3-ANT70/BSA peptide.

Serum sample	V3-ANT70/BSA coat conc. 5x	1x
NHS	0.041	0.048
R17226-5	0.038	0.052
R17226-7	0.038	0.050
R17226-24	0.041	0.052
R17226-25	0.034	0.035
R17226-68	0.090	0.084
R17226-129	0.038	0.043
R17226-136	0.048	0.046

Tabel 3. Evaluation of the V3-ANT70 ELISA with 182 human sera.

Virus strain	(sub)type tested	Nr.	V3-ANT70/BSA assay optical density ratio[a]
HIV-1 "O"	ND[b]	14	1.5 - 9.7
HIV-1 "M"	A	30	0.2 - 0.3
	B	73	0.2 - 0.6
	C	4	0.2 - 0.3
	D	6	0.2 - 0.3
	E	1	0.3
	F	2	0.2 - 0.3
	G	2	0.2 - 0.3
	H	1	0.3
	ND	16	0.2 - 0.3
HIV-2	ND	10	0.3 - 0.4
Discordant	-	13	0.3 - 0.9
HIV neg.	-	10	0.3

[a] Calculated as optical density measured/cut off value (COV). COV = 0.200.
[b] ND = not determined

Example 4

Clinical evaluation of the V3-ANT70/BSA peptide

In order to demonstrate the clinical relevance of the HIV-1 group O specific ELISA based on the V3-ANT70/BSA antigen, 182 human sera containing confirmed HIV-1 group O antibodies (14 samples), HIV-1 group M antibodies (135 samples), HIV-2 antibodies (10 samples) and as negative control normal human antibodies (10 samples) were tested. Among the 182 serum samples tested 13 samples had been classified as discordant since these serum samples had been previously shown to be positive in an ELISA based on the cyclic V3-loop region of ANT70 but negative in Western blot. All samples had been frozen in aliquots and thawed at least once and three times at the most. The subtypes of the homologous viruses of the corresponding 135 HIV-1 group M sera were determined by either sequencing or by the heteroduplex mobility assay (HMA). Out of 14 HIV-1 group O sera, the genetic characterization of the homologous viruses of 9 HIV-1 group O sera was determined by sequencing.

In the HIV-1 group O specific ELISA, all 14 HIV-1 group O sera were found reactive. By none of the remaining 168 serum samples an optical density ratio was detected higher than 0.9 (Table 3). This indicates that both the specificity and sensitivity of the HIV-1 group O specific ELISA is maximal (100%)!

Furthermore, 500 serum samples collected from HIV high risk patients of Tanzania i.e. prostitutes and truck drivers, were tested in both the HIV Uniform® II plus O kit (Organon Teknika, Belgium) as well as in the HIV-1 group O specific ELISA (data not shown). However, 33.2% of the serum samples tested was positive in the HIV Uniform® II plus O kit (Organon Teknika, Belgium). Therefore, the HIV-1 group O specific ELISA based on the V3-ANT70 peptide can be used as an HIV-1 group O specific confirmatory assay.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: AKZO NOBEL N.V.
(B) STREET: Velperweg 76
(C) CITY: Arnhem
(D) STATE: Gelderland
(E) COUNTRY: The Netherlands
(F) POSTAL CODE (ZIP): 6824 BM

(ii) TITLE OF INVENTION:
Specific HIV-1 group O antigens

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ. ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human immunodeficiency virus type 1
(B) STRAIN: group O
(C) INDIVIDUAL ISOLATE: ant70

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO: 1:

Cys Glu Arg Pro Gln Ile Asp Ile Gln Glu
1 5 10
Met Arg Ile Gly Pro Met Ala Trp Tyr Ser
15 20
Met Gly Ile Gly Gly Thr Ala Gly Asn Ser
25 30
Ser Arg Ala

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 99 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human immunodeficiency virus type 1
(B) STRAIN: group O
(C) INDIVIDUAL ISOLATE: ant70

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TGTGAAAGAC CACAAATAGA CATACAAGAG ATGAGAATAG GTCCAATGGC CTGGTACAGC 60

ATGGGAATAG GGGGAACAGC AGGAAACAGC TCAAGGGCA 99

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human immunodeficiency virus type 1
(B) STRAIN: group O
(C) INDIVIDUAL ISOLATE: mvp5180

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Cys Ile Arg Glu Gly Ile Ala Glu Val Gln
1 5 10
Asp Ile Tyr Thr Gly Pro Met Arg Trp Arg
15 20
Ser Met Thr Leu Lys Arg Ser Asn Asn Thr
25 30
Ser Pro Arg Ser Arg Val
35

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 108 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human immunodeficiency virus type 1
(B) STRAIN: group O
(C) INDIVIDUAL ISOLATE: mvp5180

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGCATAAGAG AAGGAATTGC AGAGGTACAA GATATATATA CAGGTCCAAT GAGATGGCGC 60

AGTATGACAC TTAAAAGAAG TAACAATACA TCACCAAGAT CAAGGGTA 108

Claims:

1. A peptide, selectively immunoreactive with antibodies to human immunodeficiency virus type 1 (HIV-1) group O, characterized in that said peptide comprises at least part of the amino acid sequence of a linearized V3-loop region or a functional variant thereof.

2. A peptide according to claim 1, characterized in that said peptide comprises at least part of the V3-loop region of the HIV-ANT70 isolate, or a functional variant thereof.

3. A peptide according to any of claims 1-2, characterized in that said peptide comprises at least part of the amino acid sequence shown in SEQ ID No.: 1, or a functional variant thereof.

4. A nucleic acid sequence, characterized in that said nucleic acid sequence encodes at least part of a peptide according to any of claims 1-3.

5. A nucleic acid sequence, characterized in that said nucleic acid sequence comprises at least part of the sequence as shown in SEQ ID No.: 2.

6. A recombinant vector molecule comprising a nucleic acid sequence according to any of claims 4-5.

7. A recombinant vector molecule according to claim 6, characterized in that the nucleic acid sequence is operably linked to expression control sequences.

8. A host cell transformed with a nucleic acid sequence according to any of claims 4-5 or with a recombinant vector molecule according to any of claims 6-7.

9. A process for expressing a peptide according to any of claims 1-3, characterized in that said process comprises culturing a host cell according to claim 8

10. An antibody immunoreactive with a peptide according to any of claims 1-3.

11. An immunochemical reagent comprising a peptide according to any of claims 1-3.

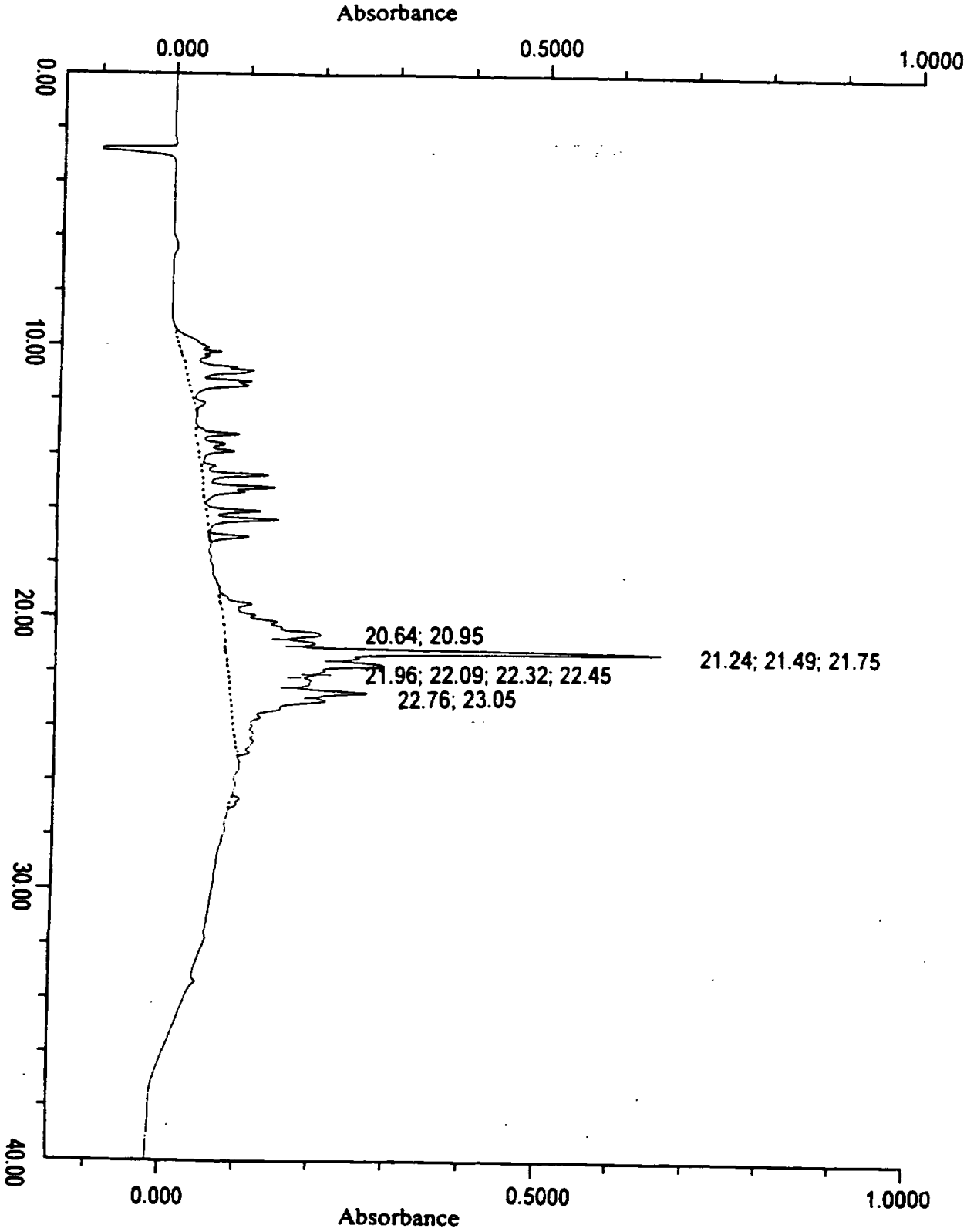
12. A method for the detection of antibodies to human immunodeficiency virus type 1 (HIV-1) group O in a sample comprising:
- contacting the sample with an immunochemical reagent according to claim 11 and
- detecting immune-complexes formed.

13. A diagnostic test kit for the detection of human immunodeficiency virus type 1 (HIV-1) group O, characterized in that said testkit comprises an immunochemical reagent according to claim 11.

14. A vaccine for the protection against human immunodeficiency virus type 1 (HIV-1) group O, characterized in that said vaccine comprises a host cell according to

claim 8 or a peptide according to any of claims 1-3, together with a pharmaceutical acceptable carrier.

15. Use of a peptide according to any of claims 1-3 for the selective detection of antibodies to human immunodeficiency virus type 1 (HIV-1) group O.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1.0000
0.00
10.00
20.00
30.00
40.00
20.64; 20.95
21.24; 21.49; 21.75
21.96; 22.09; 22.32; 22.45
22.76; 23.05
0.000
0.5000
1.0000
Absorbance

Fig. 1

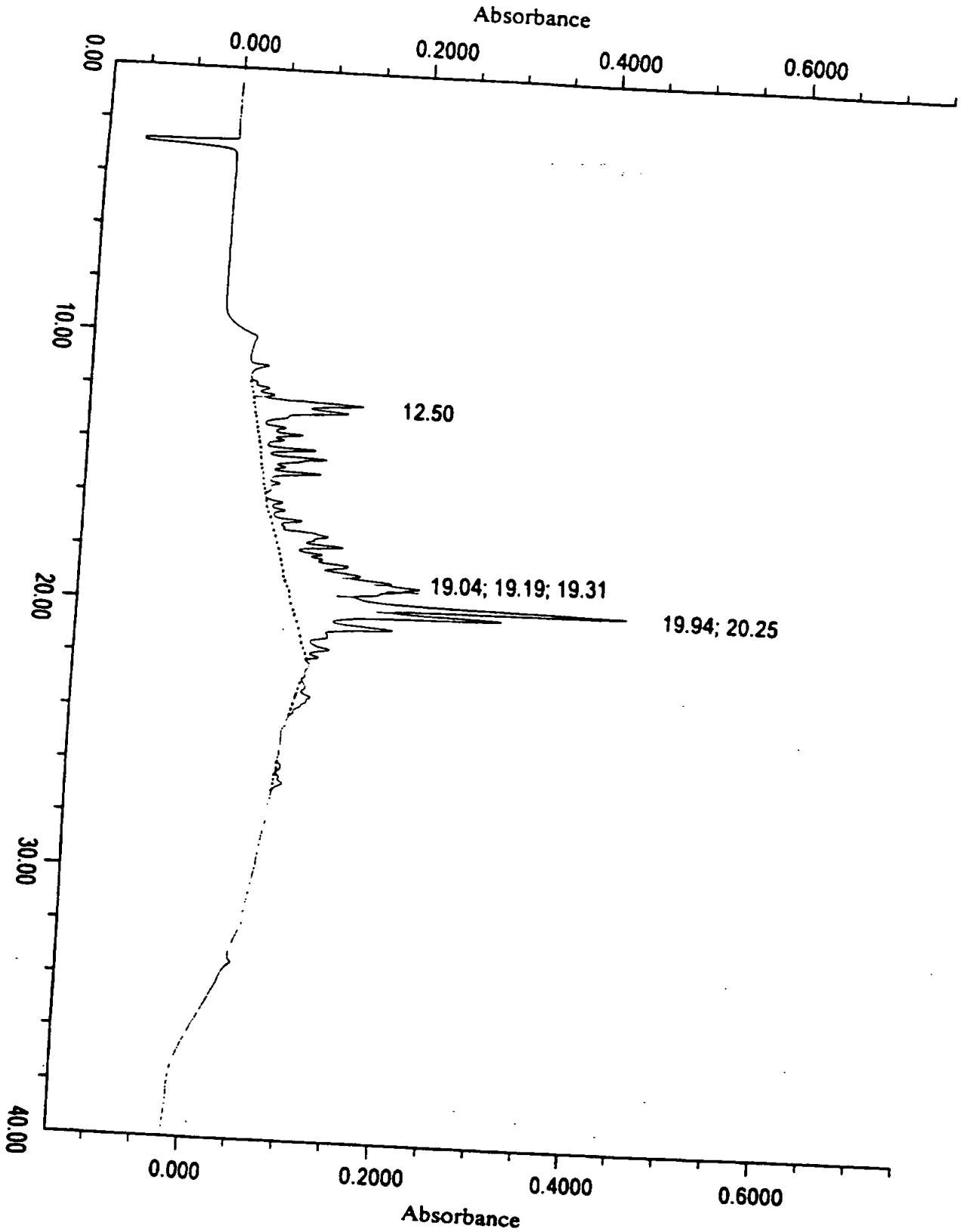
Absorbance
0.000
0.2000
0.4000
0.6000
0.00
10.00
20.00
30.00
40.00
12.50
19.04; 19.19; 19.31
19.94; 20.25
0.000
0.2000
0.4000
0.6000
Absorbance

Fig. 2

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/EP 96/00848

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/49 C07K14/16 C07K16/10 G01N33/569 A61K39/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 18054 (N.V INNOGENETICS S.A.) 16 September 1993 see sequence 9 on page 9 ---	1-3
X	JOURNAL OF VIROLOGY, vol. 68, no. 3, pages 1586-1596, XP002006620 VANDEN HAESEVELDE ET AL.: "Genomic cloning and complete sequence analysis of a highly divergent African Human Immunodeficiency Virus isolate" see the whole document --- -/--	4-9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
26 June 1996	09.07.96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	**Authorized officer** Cupido, M

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INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/EP 96/00848

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AIDS(US), vol. 8, no. 8, pages 1089-1096, XP002005315 NKENGASONG ET AL.: "Cross-neutralising antibodies to HIV-1ANT70 and HIV-1IIIB in sera of African and Belgian HIV-1-infected individuals" see page 1094, paragraph 1; figure 1	1-3,10
X	EP,A,0 591 914 (BEHRINGWERKE AG) 13 April 1994 see example 13	1,10
X	VIROLOGY, vol. 205, no. 11, 15 November 1994, ORLANDO US, pages 247-253, XP002005314 CHARNEAU ET AL.: "Isolation and envelope sequence of a highly divergent HIV-1 isolate: Definition of a new HIV-1 group" see the whole document	1,10

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 96/00848

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9318054	16-09-93	BR-A-	9305435	27-12-94
		CA-A-	2102301	07-09-93
		EP-A-	0589004	30-03-94
		JP-T-	6505806	30-06-94
EP-A-0591914	13-04-94	DE-A-	4233646	07-04-94
		DE-A-	4235718	28-04-94
		DE-A-	4244541	07-07-94
		DE-A-	4318186	05-01-95
		AU-B-	4880093	21-04-94
		CA-A-	2107732	07-04-94
		JP-A-	6225760	16-08-94
		ZA-A-	9307371	29-04-94